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Complement component C4-like protein in Atlantic cod (*Gadus morhua* L.)

- Detection in ontogeny and identification of post-translational deimination in serum and extracellular vesicles

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Abstract

The complement system is a critical part of teleost immune defences, with complement component C4 forming part of the classical and lectin pathways. Cod C4-like protein was isolated from plasma, specific antibodies generated and C4-like protein was assessed in cod sera, mucus and in extracellular vesicles (EVs) from serum and mucus. Higher levels of C4-like protein were detected in serum- than mucus-derived EVs. Post-translational deimination, caused by conversion of arginine into citrulline, can affect protein structure and function. Here we detected deiminated forms of C4-like protein in cod serum and at lower levels in mucus. C4-like protein was also found in deiminated form at low levels in EVs from both serum and mucus. C4-like protein protein was assessed by immunohistochemistry in cod larvae and detected in a range of organs including in liver, kidney, gut, muscle, skin and mucus, as well as in neuronal tissues of the brain, spinal cord and eye. This abundance of C4-like protein during early development may indicate roles in tissue remodelling, in addition to immune defences. The presence of deiminated C4-like protein in serum and mucosa, as well as in EVs, may suggest C4 protein moonlighting via post-translational deimination.

Highlights

- Complement component C4-like protein is described for the first time in cod
- C4-like protein is detected in serum, mucus, extracellular vesicles and a range of cod larval organs
- Post-translational deimination of C4-like protein differs between serum and mucus
- C4-like protein is exported in extracellular vesicles of serum and mucus at low levels
- C4-like protein is exported in deiminated forms in EVs at low levels

Key words**:** complement C4; protein deimination; extracellular vesicles (EVs); ontogeny; cod (*Gadus morhua* L.).

Introduction

The complement system forms part of the first lines of immune defences against invading pathogens and participates in the clearance of necrotic and apoptotic cells (Dodds and Law, 1998; Fishelson et al., 2001; Hart et al., 2004; Carrol and Sim, 2011). Diverse roles of complement also include regeneration (Del-Rio-Tsonis et al., 1998; Haynes et al., 2013) and tissue remodelling during development (Lange et al., 2004a; 2004b; Lange et al., 2005; Lange et al., 2006). The complement pathway can be activated via the classical, alternative or lectin pathways, with all three pathways converging to form the C3 convertase, the downstream lytic pathway and the membrane attack complex (MAC), leading to killing of the microorganism (Dodds and Law, 1998; Sunyer and Lambris, 1998; Volanakis, 2002; Dodds, 2002). The classical pathway is activated either via direct binding of C1 to acute phase proteins or proteins of bacterial and viral origin, or via the C1q subcomponent which can also bind to the Fc region of immunoglobulins that are bound to antigen (Reid et al., 2002; Reid, 2018). Downstream of C1 activation, C4 is cleaved and contributes to the formation of the C3 convertase of the classical pathway. While C3 has previously been described in cod (Lange et al., 2004a; 2004b; 2004c; 2005), C4 has hitherto not been studied in cod.

Complement component C4 is a thioester containing glycoprotein composed of three disulphide linked chains; α-, β- and γ-chains, which in human are 94, 72, and 30 kDa respectively, and is found in two isotypes which differ in the presence of a catalytic histidine (Dodds et al., 1996). The presence of the two C4 isotypes has been verified in various jawed vertebrates including sharks, reptiles and birds (Nonaka et al., 2017). In teleost fish, the structural and functional diversification of complement components has received considerable attention and is believed to contribute to balancing inflammatory and homeostatic processes (Sunyer and Lambris, 1998; Kuroda et al., 2000; Boshra et al., 2004; Boshra et al., 2006; Nakao et al., 2011).

Previous studies from our group include ontogeny studies on complement component C3 in early teleost development (Magnadottir et al., 2004; Magnadottir & Lange 2004; Magnadottir et al., 2005; Lange et al., 2004a; Lange et al., 2004b; Lange et al., 2005, Lange et al., 2006; Magnadottir et al., 2006) and on post-translationally deiminated proteins in teleost ontogeny and immunity (Magnadottir et al., 2018a and 2018b; Magnadottir et al., 2019a). Furthermore, recent work from our group has focussed particularily on extracellular vesicles and protein deimination both in teleost and elasmobranch species (Magnadottir et al., 2019b and 2019c; Criscitiello et al., 2019).

Extracellular vesicles (EVs) are small lipid vesicles (30-1000 nm) which are released from cells and play roles in cell communication via transfer of a range of protein and genetic cargo. EVs are present in most body fluids and are widely studied in human health and disease, including for development

as biomarkers (Inal et al., 2013; Hessvik and Llorente, 2018). EV research is an emerging field in fish immunology, also for the development of usable biomarkers to assess fish health in response to environmental and immunological challenge (Iliev et al., 2018; Lagos et al., 2017; Magnadottir et al., 2019b and 2019c).

Post-translational deimination is caused by peptidylarginine deiminases (PADs), a phylogenetically conserved group of calcium catalysed enzymes which cause post-translational conversion of arginine into citrulline (Vossenaar et al., 2003; György et al., 2006). This causes structural, and sometimes functional, changes in target proteins (Witalison et al., 2015) and may also allow for protein moonlighting, facilitating proteins to take on a range of functions within one polypeptide chain (Jeffrey, 2018). We recently described novel roles for protein deimination in mucosal and innate immunity of teleost fish, including deimination of key immune factors (Magnadottir et al., 2018a; 2018b; 2019a; 2019b; 2019c).

In the current study we set out to detect complement component C4 protein in early cod larval development, to assess putative post-translational deimination of C4 in cod serum and mucus and to confirm whether C4 forms part of EV cargo in serum and mucus. The findings of this study may shed new light on functional diversity of C4-like protein in cod.

2. Materials and Methods

2.1 Fish and sampling

Experimentally farmed adult cod (*Gadus morhua* L) and larvae were obtained from the Marine Institute's Experimental Fish farm Stadur, Grindavik, Iceland; reared as described before (Lange et al., 2004a; Magnadottir et al., 2018a). For isolation of C4, fish blood (5-10 ml) was collected into ethylene-diamine-tetra-acetic (EDTA)-coated syringes (Monovette, Germany) from the caudal vein of 20 adult cod. The blood was placed on ice for 2 h and plasma was collected by centrifuging at 750 *g* for 10 min. The samples from individual fish were pooled and stored at 4 °C in the presence of 5 mM EDTA until used. Human citrated plasma was obtained from HD Supplies (Aylesbury, U.K.) and EDTA added to a final concentration of 10 mM before use. For preparation of sera, collected blood was allowed to clot overnight at 4 °C and serum was collected after centrifuging at 750 *g* for 10 min, divided into aliquots and stored at -80 °C until used. Mucus was collected and prepared from adult cod as previously described (Magnadottir et al., 2018a). Cod larvae were collected during the spawning season (April-June) as previously described (Lange et al., 2004a), and for this study two age stages, 28 and 57 days post hatching (d.p.h), were used to assess C4 protein detection in tissues and

organs, using longitudinal sections through the whole larvae. Four larvae were used for each sampling date, fixed in 4 % formalin in phosphate buffered saline (PBS) at 4 °C for 24 h and thereafter embedded in paraffin and stored at room temperature until used.

2.2 Cod C4 isolation and antibody generation

Human C4 was prepared as described by Dodds (1993) and used as a standard during the purification of cod C4 protein, which was purified using the same method. C4-like protein was isolated from cod plasma by column chromatography using Q-Sepharose HP, followed by MonoQ and Superdex 200 (HR 10/30) (Amersham Biosciences) gelfiltration columns, after initial precipitation by 5 % PEG (polyethylene glycol) (Dodds, 1993). Purified cod C4-like protein fractions (cod-C4) were compared to human C4 (Hu-C4) and separated under reducing conditions respectively on a 10 % polyacrylamide gel and Coomassie blue stained (Fig. 1A). The purified fractions were subjected to N-terminal sequence analysis using cut-out bands (performed in-house by Anthony Willis, MRC Immunohistochemistry Unit Oxford), using the Applied Biosystems 470A/120A protein sequencer as previously described (Crawford et al., 1990). N-terminal analysis of both C4α- and β-chains from cod C4-like protein was compared to other species, using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and protein sequences from human (*Homo sapiens*; AAB59537.1), Xenopus (*Xenous laevis*; AAI70420.1), nurse shark (*Ginglymostoma cirratum*; AAY55950.1), channel catfish (*Ictalurus punctatus*; XP_017326706.1), carp (*Cyprinius carpio*; XP_018973374.1, BAB03284.1) and Japanese medaka (*Oryzias latipes*; NP_001098167.1) (Fig. 1B).

Polyclonal anti-cod C4-protein like antibodies were produced in mouse ascetic fluid according to the method of Overkamp et al. (1988), as previously described for cod C3 (Lange et al., 2004c). Ascetic fluid was collected, aliquoted and stored at -80 °C. The specificity of the anti-cod C4-protein like antibody was tested on purified cod C4 (Fig. 1B) and on cod serum (Fig. 2A).

2.3 Extracellular vesicle isolation from mucus and serum

EVs were isolated from cod sera and mucus using step-wise centrifugation. First, serum and mucus were diluted 1:4 and 1:5 respectively, in sterile filtered Dulbecco's phosphobuffered saline (DPBS), adding 250 µl serum to 750 µl DPBS, or 200 µl of mucus to 800 µl DPBS, respectively. These were centrifuged at 4,000 *g* for 30 min at 4 °C to remove cell debris and aggregates, and thereafter the supernatant was collected and ultracentrifuged at 100,000 *g* for 1 h at 4 °C. The obtained EV pellets were resuspended and washed in sterile DPBS, ultracentrifuged again at 100,000 *g* for 1 h at 4 °C and the resulting EV pellets were solubilised in 50 µl DPBS and subjected to nanoparticle tracking analysis (NTA) and protein extraction for immunoprecipitation and for Western blotting.

2.4 Characterisation of EVs

EVs were characterised using NTA, Western blotting and transmission electron microscopy (TEM). For NTA analysis, the individual EV pellets derived from 250 µl of serum or 200 µl of mucus respectively, per sample, were resuspended in 50 µl DPBS and diluted 1/200 in DPBS. EVs were assessed for size distribution based on Brownian motion, using the NanoSight NS300 (Malvern, U.K.). Samples were applied to the NanoSight using a syringe pump to ensure even flow of the sample, with numbers of particles in the window at 40 - 60 per frame. Videos were recorded for 5 x 60 sec and the histograms generated from the individual repetitive reads were averaged. EVs were tested by Western blotting for CD63 and Flotillin-1, which are EV-specific markers conserved in bony fish (Iliev et al., 2018). For TEM, EVs were fixed with 2.5 % glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.0) for 1 h at 4 °C, resuspended in 100 mM sodium cacodylate buffer (pH 7.0), placed on to a grid with a glow discharged carbon support film and stained with 2 % aqueous Uranyl Acetate (Sigma-Aldrich) (Supplementary Fig. 1).

2.5 Immunoprecipitation

Total deiminated proteins were isolated by immunoprecipitation from a pool of sera, a pool of mucus protein extract, or EVs from both, respectively. Immunoprecipitation was performed using the Catch and Release® v2.0 Reversible Immunoprecipitation System (Merck, U.K.) according to the manufacturer's instructions, and the monoclonal F95 pan-deimination antibody (MABN328, Merck), which specifically recognises deiminated proteins (Nicholas and Whitaker, 2002). F95-bound proteins were eluted under reducing conditions, according to the manufacturer's instructions, and diluted 1:1 in 2x Laemmli sample buffer for Western blotting analysis.

2.6 Western blotting

Protein samples prepared from cod C4-like protein fractions, sera, mucus, EVs and the corresponding F95-bound eluates were analysed by Western blotting for detection of C4-like protein. Approximately 5 µg of protein sample was loaded per lane, even load was assessed using Ponceau S staining (Sigma, U.K.), membranes were thereafter blocked in 5 % bovine serum albumin (BSA) in Tris buffered saline with 0.1% Tween20 (TBS-T) for 1 h, followed by incubation at 4 °C overnight with the primary anti-C4 protein-like antibody (1/1000; in TBS-T). EV specific markers used for EV characterisation were CD63 (ab216130, 1/1000) and Flot-1 (ab41927, 1/2000). Following primary antibody incubation, membranes were washed 3 times in TBS-T, incubated at room temperature for 1 h with HRP-conjugated secondary antibodies (anti-mouse IgG or anti-rabbit IgG; BioRad, U.K.), followed by 6 washes in TBS-T before visualisation using ECL (Amersham, U.K.) and the

UVP BioDoc-ITTM System (U.K.). For Fig. 1, Western blots were developed using alkaline phosphatase conjugated secondary mouse-IgG (Dako, Denmark) and 0.1 M ethanolamine/HCl buffer (pH 9.6) containing NBT (1 mg ml−1p-nitroblue tetrazolium), 0.1 M $MgCl_2$ and BCIP (4 mg ml−15-bromo-4chloro-indolyl phosphate in methanol:acetone (2:1).

2.7 Immunohistochemistry

For immunohistochemistry, 5 micron serial tissue sections were cut on a microtome to detect the presence of C4-like protein in cod larvae at two developmental stages, at 28 d.p.h., for an earlier stage, and 57 d.p.h. when cod larvae have reached immunocompetence (Magnadottir et al., 2005). Tissue sections were deparaffinised using xylene, taken to water (100%, 90%, 70% ethanol) and de-masked by heating (12.5 min in the microwave at power 8) in citric acid buffer (pH 6.0). Thereafter the sections were washed in 0.1 % BSA in 100 mM phosphate buffer (PB) and washed two times in PB. Next the sections were incubated with 5 % goat serum (Sigma, St. Louis, MO, USA) in PB for 1 h, followed by incubation in the anti-cod C4 primary antibody, diluted 1/100 in PB/BSA at 4 °C overnight. The sections were thereafter washed for 2 min in serial washes of PB/BSA, PB, PB and PB/BSA, and incubated with biotin-labelled anti-mouse IgG (1/200; Vector Laboratories, Inc., Burlingame, CA, USA). Visualisation was performed using Avidin-Biotinylated peroxidase Complex (ABC, Vector Laboratories, Inc.) and diaminobenzidine/hydrogen peroxide (DAB) stain (Vector Laboratories). Sections were counterstained with Mayer's Haematoxylin (Sigma, U.K.), dehydrated in alcohol, immersed in xylene and mounted with DEPEX (Sigma). Four larvae for each developmental stage were analysed.

3. Results

3.1 C4 isolation and antibody generation

Cod C4-like protein was isolated from 20 ml of pooled EDTA plasma, using PEG precipitation and purification on Q-Sepharose HP, followed by MonoQ and Superdex 200 (HR 10/30) gelfiltration columns. The purified cod C4 fractions were analysed by SDS-PAGE and Coomassie blue staining and found to have a three chain structure, similar to human C4 (Fig. 1A). For purified cod C4-like protein, the approximately 90 and 68 kDa bands are consistent with the C4 α- and β-chains respectively, while the band below 37 kDa is indicative of the γ-chain. The 250 kDa band corresponds to PRO-C4, i.e. the form of C4 that has not been cleaved to form 3 chains. Other bands may represent α-chain fragments (α-f), including smaller bands below the β-chain. The 250 kDa band corresponds to PRO-C4, i.e. the form of C4 that has not been cleaved to form 3 chains. Other bands may represent alpha

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chain fragments, including smaller bands below the β-chain. N-terminal amino acid sequencing was performed of both the C4α- and β- chains and the resulting sequences were aligned with corresponding sequences from other species throughout phylogeny (Fig. 1B). The C4 sequences from the following species are shown for comparison: human (*Homo sapiens*; AAB59537.1), Xenopus (*Xenous laevis*; AAI70420.1), nurse shark (*Ginglymostoma cirratum* AAY55950.1), channel catfish (*Ictalurus punctatus*; XP_017326706.1), carp (*Cyprinius carpio*; XP_018973374.1, BAB03284.1) and Japanese medaka (*Oryzias latipes*; NP_001098167.1) (Fig. 1B). Polyclonal antibodies, produced against the whole cod C4-like protein in mouse ascetic fluid, were confirmed to react with the purified C4 fractions (Fig. 1C) and showed positive for all three C4 chains (α-, β- and γ-chains), as well as α-chain fragments (α-f), in cod serum and mucosa (Fig. 2B).

3.2 C4 detection in serum, mucus and extracellular vesicles (EVs).

Cod C4-like protein was detected in serum and mucus, at similar levels (Fig. 2A). EVs isolated from cod serum and mucus (Supplementary Fig 1) were assessed for C4 cargo. In serum-derived EVs, C4-like protein was found at higher levels compared to EVs isolated from mucus (Fig. 2A).

3.3 C4-like protein deimination varies between serum and mucus

To identify if C4-like protein was post-translationally deiminated, the F95-bound eluates, from serum and mucus respectively, were probed with the anti-cod C4-like protein antibody. The C4-like protein antibody was found to react with the F95 eluate of both serum (Fig. 2B) and mucus (Fig. 2C), albeit with some differences. In serum, bands representative of C4 were stronger deimination positive, while in mucus only very low levels of C4-like protein showed deimination positive. In EVs derived from serum and mucus, the F95 eluate showed some low positive for C4 (Fig. 2D).

3.4. C4 immunohistochemistry in cod larvae

Using immunohistochemistry, longitudinal tissue sections of larval stages of cod aged 28 and 57 days post hatching (d.p.h.) were assessed for tissue distribution of C4-like protein. A similar pattern of C4-like protein detection was found at these two stages of development. C4-like protein was detectable in immune organs including liver, where hepatocytes were strongly C4 positive (Fig. 3A), and in kidney, where both glomeruli and tubuli were strongly C4-like protein positive (Fig. 3B). In gut, C4-like protein was strongly detected in mucosa (Fig. 3C), in gill mucosa and chondrocytes (not shown), as well as in skin mucosa and in muscle (Fig. 3D). C4-like protein was also seen in mucosa of the mouth and oesophagus (not shown) as well as in chondrocytes (not shown). C4-like protein was clearly detected in neuronal tissues including in brain (Fig. 3E) and spinal cord (not shown) as well as

in the eye, with C4-like protein positive cells in the inner and outer ganglion layers, and plexiform layers (Fig. 3F).

4. Discussion

Complement component C4-like protein is here described for the first time in Atlantic cod (*Gadus morhua* L.). C4-like protein was found in serum and mucus at similar levels, indicating roles in the immune defence at these sites. Extracellular vesicles (EVs) were assessed for C4 protein cargo, which was more abundant in EVs from sera than mucus. For the first time, deiminated forms of C4-like protein were identified in cod serum and mucus. Post-translationally deiminated C4-like protein was detected in serum and mucus and C4-like protein was found to be exported in deiminated forms at low levels in both serum and mucus derived EVs. In comparison, C3 was recently reported at high levels in EVs from both mucus and serum, including in deiminated form (Magnadottir et al., 2019b and 2019c), indicating marked differences in EV-mediated export of these two complement components.

Post-translational deimination is a means of increasing antigenic diversity via changes in protein structure and folding, leading to altered antigen processing, antigen presentation and immune recognition (Doyle and Mamula, 2012). These changes can for example alter interaction with immune cells and also affect signalling pathways (Nguyen and James, 2016). Deimination of C4-like protein, observed here in cod serum and mucus, may indicate a mode for functional diversity of C4 at these sites, and possibly influence its ability for cleavage and formation of the classical pathway C3 convertase. Indeed, pharmacological inhibitors of deimination have been shown to ameliorate collagen-induced arthritis via decreased complement deposition in synovium and cartilage (Willis et al., 2011; Willis et al., 2017). A similar phenomenon of post-translational deimination of C4 as seen here in cod C4-like protein, was recently identified in a range of complement factors, including C3 and C4 (Magnadottir et al., 2019a).

Here we report C4-like protein detection in a range of immune-related, neuronal and mucosal tissues in early cod ontogeny. This is a similar pattern as previously observed for complement component C3 in cod larvae (Lange et al., 2004a; 2005). C4 protein was high in liver, which is the main site of complement synthesis, as well as in a range of other organs. C4 was strongly detected in kidney, both in tubuli as well as in glomeruli, which are known sites of complement component synthesis (Morgan and Gasque, 1997) and also previously shown to synthesise C3 in cod (Lange et al., 2004a; 2005). C4 was strongly detected in mucosal surfaces of the skin, gut, as well as in gills. This is in line with the importance of the alimentary tract, gut, gills and mucosal surfaces on the epidermis for fish immune defences (Ellis, 2001; Parra et al., 2015; Gomez et al., 2013). Intestinal

cells are known to be local production sites for complement components, including C4 (Andoh et al., 1993; Laufer et al., 2000). In neuronal tissue, C4 protein was seen at high levels in spinal cord, brain and eye, similar to as previously observed for cod C3 (Lange et al., 2004a; 2005), and may indicate roles in tissue remodelling during ongoing neurogenesis at these sites (Barnum et al., 1995; Morgan and Gasque, 1996; Terai et al., 1997). As the complement system participates in apoptotic processes, phagocyte recruitment and opsonisation of apoptotic cells (Fishelson et al., 2001; Hart et al., 2004), C4 may contribute to homeostatic roles of the complement system in tissue remodelling during development (Cole and Morgan, 2003; Lange et al., 2005; Magnadottir et al., 2019a). Furthermore, C4-like protein deimination in mucus and serum suggests temporal and spatial changes in C4 post-translational modification to facilitate diverse functions of C4, according to environmental requirements. In comparison, halibut C4 was also recently identified to be deiminated in serum (Magnadottir et al., 2019a), further indicating functional diversity of C4 via deimination which remains to be further investigated throughout phylogeny.

The findings presented in the current study provide novel insights into putative moonlighting functions of C4 in immunity, during development and in the maintenance of homeostasis.

Conclusions

Complement component C4-like protein is here described for the first time in Atlantic cod (*Gadus morhua* L.) and found to be post-translationally deiminated in mucus and serum, indicative of functional diversity. Extracellular vesicles (EVs) were found to contain C4 protein cargo, which was more abundant in EVs from sera than mucus. Furthermore, deiminated C4 was also observed in EVs at low levels. C4-like protein was detected in early cod ontogeny in a range of organs, indicative of roles in immune defences and tissue remodelling during larval development. C4 may, through post-translational deimination, take on diverse functions to meet temporal and spatial requirements in host defence and maintenance of homeostasis.

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Figure legends.

Fig. 1. C4 isolation from cod plasma. A. Purified cod C4 fractions isolated from cod plasma (cod-C4) by column chromatography are shown compared to purified human C4 isolated from human plasma (Hu-C4) and separated under reducing conditions on a 10 % polyacrylamide gel and Coomassie blue stained. The C4α-, β-, and γ- chains are indicated. The approximately 90 and 68 kDa bands are consistent with the C4 α- and β-chains respectively, while the band below 37 kDa is indicative of the γ-chain. The 250 kDa band corresponds to PRO-C4, i.e. the form of C4 that has not been cleaved to form 3 chains. Other bands may represent α-chain fragments (α-f), including smaller bands below the β-chain. **B.** The purified C4 fractions were assessed by N-terminal analysis and compared to C4 from other species to verify identification of cod C4. Conserved amino acids throughout phylogeny are underlined and phylogenetic clustering for the C4-like protein α- and β-chains is shown. Sequences used were: human (*Homo sapiens*; AAB59537.1), Xenopus *(Xenous laevis*; AAI70420.1), nurse shark (*Ginglymostoma cirratum* AAY55950.1), channel catfish (*Ictalurus punctatus*; XP_017326706.1), carp (*Cyprinius carpio*; XP_018973374.1, BAB03284.1) and Japanese medaka (*Oryzias latipes*; NP_001098167.1, XP_024115560.1). **C.** Polyclonal mouse anti-cod C4-like protein antibody was produced and tested on the C4-like protein fractions.

Fig. 2. C4 detection in serum and mucus and verification of deiminated C4. **A.** Western blotting showing C4 in serum, mucus and in EVs isolated from serum and mucus, respectively. C4 is strongly detected in both serum and mucus, and at higher levels in EVs of serum than mucus. The C4 α- β- and γ-chains are indicated as well as α-chain fragment (α-f). **B.** Deiminated C4 is detected in serum, by assessing the F95-enriched eluate (which was obtained by IP from cod serum using the pan-deimination F95 antibody) against the C4 antibody. The C4 β- and γ-chains are indicated. **C.** Deiminated C4 is hardly detectable in mucus, by assessing the F95-bound eluate against the C4 antibody. A faint band representative of the C4 β-chain is visible as well as a very faint band in the γ-chain region, but at much lower levels than what is seen in serum (see B). **D.** Deiminated C4 was detected at very low levels in EVs from serum and mucus, by blotting the F95-bound eluate against the C4 antibody. C4 β- and γ-chains are indicated.

Fig. 3. Histology of C4-like protein in cod larvae. A. Liver (28 d.p.h.): hepatocytes are strongly C4 positive; **B.** Kidney (28 d.p.h.): glomerulus (gl) and tubuli (tub) are strongly C4 positive and indicated by arrows; **C.** Gut (28 d.p.h.): mucosal cells are strongly C4 positive; **D.** Skin (sk), skin mucosa (mu) and muscle (mus) are C4 positive (57 d.p.h.); **E.** Neurones in brain show strong C4 positive (57 d.p.h.); **F.** In eye (7 d.p.h.) the plexiform (pl), outer ganglion (og) and inner ganglion (ig) layers are strongly C4 positive. The scale bars indicate 100 µm in all figures.

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Supp. Fig. 1. **EV isolation and characterisation from serum and mucus. A.** Serum and mucosal EVs were characterised by nanoparticle tracking analysis (NTA) and a representative histogram shows a typical polydispersed population of EVs falling mainly between 30-400 nm in size; **B.** Western blotting of EVs from serum and mucus, respectively, for the EV-specific markers Flot-1 and CD63; **C.** Transmission electron microscopy (TEM) of serum and mucosal EVs (scale bar = 200 nm).

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Fig. 3

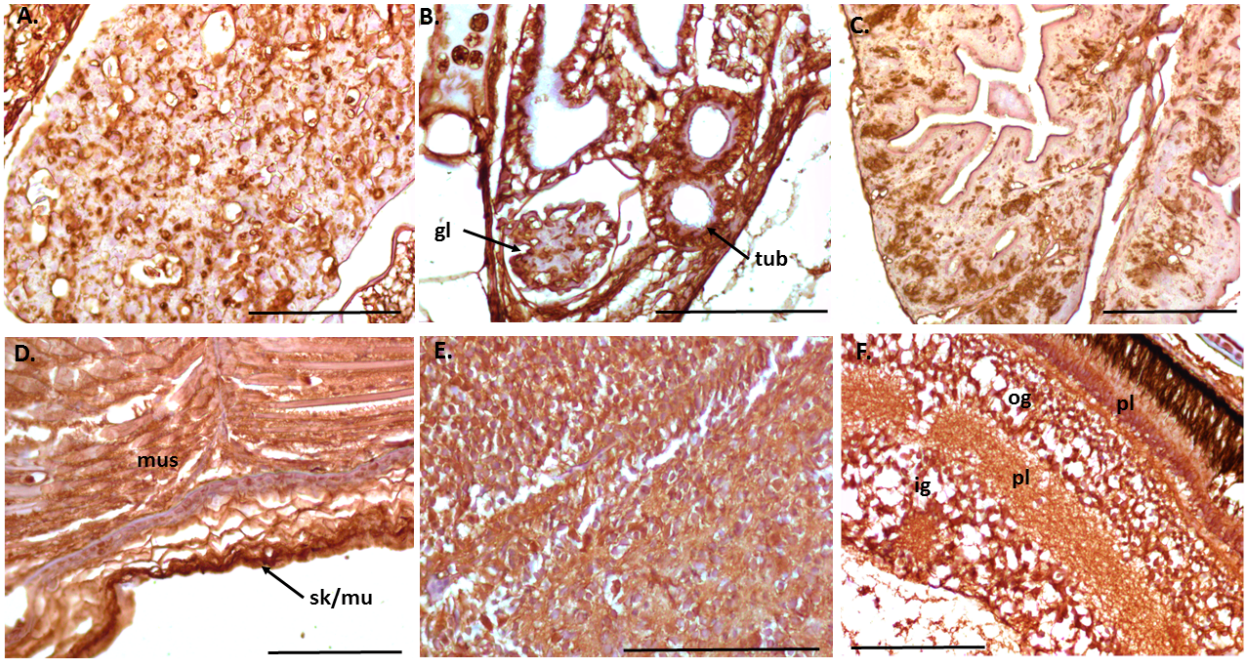

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Fig. 1

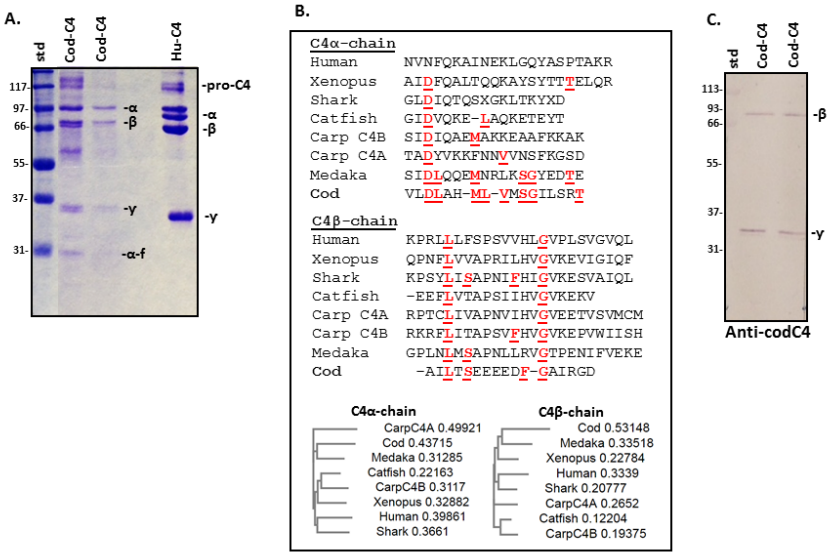
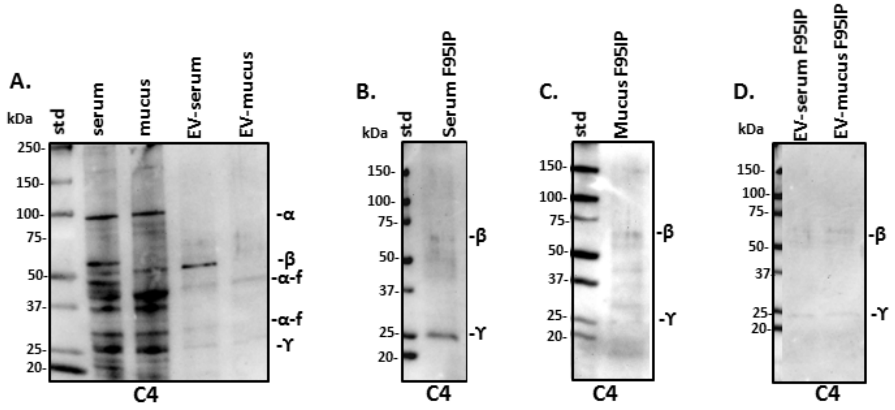


Fig. 2



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